# APPENDIX A

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# The Expression of Truncated MK in Human Tumors

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Midkine (MK) is a heparin binding growth/differentiation factor different from fibroblast growth factors (FGFs), and is largely composed of two domains which are formed by a folded polypeptide chain interconnected by disulfide bridges. Polymerase chain reaction revealed the presence of a short MK mRNA in 7 among 12 human tumor cells which expressed MK mRNA. All of 4 pancreatic carcinoma cell lines expressed the short species in addition to the full size mRNA. The short mRNA lacked an exon and resulted in the lack of a more N-terminally located domain. The truncated form of MK was found also in some surgically removed specimens of human tumors, but not in noncancerous tissue. 

Midkine (MK) is a novel growth factor found as a product of a retinoic acid responsive gene, and has neurotrophic activities (1–4). It consists of 5 exons (5) and is located on chromosome 11q11.2 in human (6). MK mRNA is strongly expressed in many human carcinoma cells (7,8). In the case of Wilms' tumor, all 6 of surgically removed specimen showed intense MK expression (7) and anti-MK antibody partly inhibited growth of cultured tumor cells (4), indicating that it plays a role in enhanced growth of Wilms' tumor cells.

Altered forms of growth factors or products of tumor suppresser genes are often produced in tumor cells by alternative splicing, and they play key roles in tumorigenesis (9).

So far it is not known whether altered MK structure is present in tumor cells or normal tissues. This paper demonstrates altered processing of MK mRNA in human tumor cells.

## MATERIALS AND METHODS

### *Tumor Cell Lines*

The three lung adenocarcinoma cell lines (A549, PC-3, RERF-LCOK), the gastric adenocarcinoma cell line (NUGC-3), the hepatoma cell line (HuH7), two lymphoma cell lines (U937, Molt4), and the Wilms' tumor cell line (G401) were obtained from the Japanese Cancer Research Resource Bank (Tokyo, Japan). The pancreas adenocarcinoma cell lines (AsPC-1, BxPC-3, Capan-1) were obtained from the American Type Culture Collection (Rockville, MD). The esophagus carcinoma cell line (TEN-8) (ref. 10) and the pancreas carcinoma cell line (SUIT-2) (ref. 11) were generously offered from Department of Surgery, Tohoku University and Department of Surgery, Miyazaki Medical College respectively. The two cell lines GaCa (12) and GBK-1 were established from human gastric adenocarcinoma and gall bladder carcinoma respectively.

### *RNA Isolation*

The human cell line G401 was cultured in McCoy 5A, 10% FCS. The other cell lines were cultured in RPMI 1640, 15% FCS, and were harvested by 0.25% trypsin-EDTA. Tumor specimens and the normal tissues were surgically removed from cancer patients. Total cellular RNA from all cell lines, tumors, and normal tissues was extracted using guanidine-thiocyanate-phenol-chloroform method (13).

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Abbreviations: MK, midkine; RT–PCR, reverse transcript–polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.



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### *Northern Blots*

Twenty μg of total RNA was denatured, electrophorased through formaldehyde gels, and transferred to a Hybond N+ nylon membrane (Amersham, UK). Full length of MK cDNA was used as the probe. Radio labeled hybridization probes were prepared with the random hexamer-labeling. Hybridized membrane was washed with 2×SSC, 0.1%SDS at 65 °C for 30 min and subjected to autoradiography.

### *cDNA Synthesis*

cDNA from total RNA was synthesized with Superscript II preamplification system (GIBCO-BRL, MD). In brief, 3 μg of total RNA was digested with RNase free-DNase, heat at 70°C for inactivation of the enzyme, and denatured and hybridized with oligo dT primers. Then 1st strand cDNA was synthesized by reverse transcriptase, and RNA was digested by RNase H. The 1st-strand cDNA was used for polymerase chain reaction (PCR) as the template.

### *PCR*

One μl aliquot of the 10-fold diluted cDNA solution was subjected to PCR in 20 μl. An oligonucleotide sequence in the sense strand of human MK cDNA (5'-ATGCAGCACCGAGGCTTCCT-3': 1-20, ref. 7), in the antisense strand of MK cDNA (5'-ATCCAGGCTTGGCGTCTAGT-3': 450-428, ref. 7), in the sense strand of human glyceraldehyde 3-phosphate dehydrogenese (GAPDH) cDNA (5'-AATCCCATCACCATCTTCCA-3': 274-293, ref. 14), and in the antisense strand of GAPDH cDNA (5'-CCAGGGGTCTTACTCCTTG-3': 1077-1059, ref. 14) were synthesized using ABI 392 DNA/RNA synthesizer and used as the primer of the PCR. The PCR conditions were as follows; 28 cycles of denaturation (94°C: 1 min); re-annealing (58°C: 2 min); and extension (72°C: 2 min). The PCR products were detected by agarose gel electrophoresis with ethidium bromide staining and Southern blot analysis.

### *DNA Sequence Analysis*

The PCR products were isolated and were subcloned into pGEM-T vector (Promega, WI). The DNA sequence was determined by cycle sequencing method using fluorescence autosequencer (373A, ABI).

## RESULTS

### *MK Expression in Various Cell-Line-Derived Tumors*

Among 15 tumor cell lines analyzed, 12 were revealed to produce the product corresponding to the expected PCR band of 450 bp. These bands were hybridized with MK cDNA probe (data not shown). However, in 7 among the 12 positive cell lines, we also detected another PCR band smaller than the expected MK band (Fig. 1). The size of the small band was about 280 bp. These bands were also hybridized with MK cDNA probe (data not shown). The smaller band was detected in 4 pancreatic carcinoma cell lines, but not in any of 3 lung carcinoma cell lines.


**FIG. 1.** Detection of truncated form of MK in cDNAs from human tumor cells by polymerase chain reaction. cDNAs were amplified by PCR using primers specific for human MK cDNA, and the products were analyzed by 2% agarose gel electrophoresis. Arrows indicate the mature form of MK cDNA and the truncated form. M, size marker; lane 1, A549 cells (lung adenocarcinoma); lane 2, PC-3 cells (lung adenocarcinoma); lane 3, RERF–LCOK cells (lung adenocarcinoma); lane 4, TEN-8 cells (squamous cell carcinoma of the esophagus); lane 5, GaCa cells (gastric adenocarcinoma); lane 6, NUGC-3 cells (gastric adenocarcinoma); lane 7, HuH 7 cells (well differentiated hepatocellular carcinoma); lane 8, GBK-1 cells (gall bladder carcinoma); lane 9, SUIT-2 cells (moderately differentiated pancreatic adenocarcinoma); lane 10, AsPC-1 cells (moderately differentiated pancreatic adenocarcinoma); lane 11, BxPC-3 cells (poorly differentiated pancreatic adenocarcinoma); lane 12, Capan-1 (well-differentiated pancreatic adenocarcinoma); lane 13, U937 myeloblasts; lane 14, Molt4 T cell leukemia cells; lane 15, negative control; lane 16, G401 cells (Wilms' tumor). The truncated band was clearly seen in lanes 6, 7, 9, 10, 11, 12 and 16.

*Sequence and Structure of the Normal Size Products and of the Smaller Products*

As a representative case, the normal size-band and the smaller band from SUIT-2 cell line were isolated, and were subcloned into pGEM-T vector. Sequence of the subcloned normal size MK-cDNA indicated that it had no mutation or deletion. The MK-cDNA derived from G401 also had no mutation or deletion. The smaller band from SUIT-2 cell line lacked exon 3 human MK (Fig. 2). All other sequence are identical to coding sequence of the wild type human MK cDNA. This truncated form of mRNA had no frame shift mutation.

*MK Expression in Non-carcinoma Tissues*

We investigated expression of MK in various normal tissues by RT-PCR (Fig. 3, 4C). In some tissues, wild type MK were expressed. But truncated form of MK was not expressed in any tissues.

*MK Expression in Surgically Removed Tumors*

We investigated 3 cases of gastric tumor, 3 cases of pancreatic tumor and a case of Wilms' tumor by RT-PCR and Northern blot analysis (Fig. 4). In all the cases, gastric carcinoma expressed normal size MK mRNA, and expressed truncated form of MK mRNA in two cases (Fig. 4A, 4D). The ratio of the normal size of MK mRNA and the truncated form was about 8:1 in a case, and 6:1 in another case as estimated by Northern blotting (Fig. 4D). In 3 cases of pancreatic tumor and a case of Wilms' tumor, we compared expression between tumor tissues and normal tissues in the same individual (Fig. 4B, 4C). Then the truncated MK mRNA was in tumor tissues but not in non-carcinoma tissues.

## DISCUSSION

Midkine is largely composed of two domains, each of which is compactly held by two or three disulfide bridges (15). Exon 3, which is deleted in the truncated mRNA, encodes the entire domain located in the N-terminal side and some adjacent amino acids (5, 16). More specifically, the peptide portion between Asp26 and Gly81 (for numbering, refer to 7) was deleted in the truncated form. Thus, the truncated form has about 55% of the size of the intact form.

Some function of MK, namely, neurite-outgrowth promoting activity and enhancing activity of

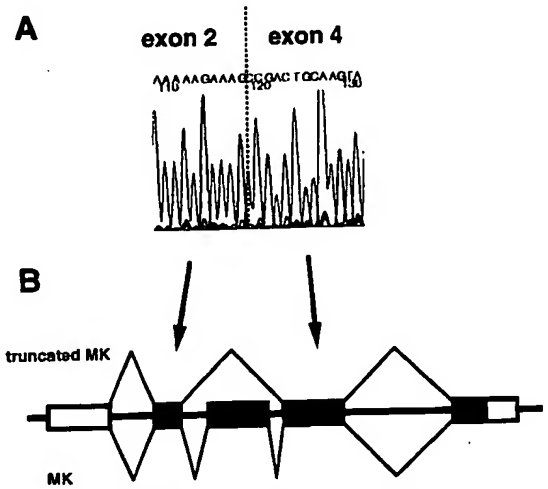


FIG. 2. cDNA sequence and splicing scheme of the truncated form of MK. A, nucleotide sequence at the junction of exon 2 and 4 in the truncated form. B, alternative splicing of the truncated MK. Exons are represented by open boxes, coding region by solid boxes, and introns by narrow lines. Splicing between the exons is indicated by thin lines. The truncated form of MK is assembled by skipping of exon 3.

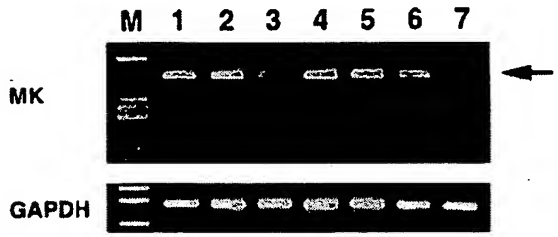


**FIG. 3.** MK expression in normal tissues. An arrow indicates the mature form of MK cDNA. Lane 1, thyroid gland; lane 2, lung; lane 3, stomach; lane 4, small intestine; lane 5, colon; lane 6, spleen; lane 7, liver. MK was not expressed in the liver.

plasminogen activator in aortic endothelial cells is located in C-terminal half of MK molecule or in the C-terminally located domain of MK-molecule (17, 18). Therefore, the product of the truncated form of MK is expected to retain certain MK function. However, because of the lack of N-terminally located domain, the truncated form of MK may lack a region that is required for their proper localization in extracellular matrices. Such disorganization can result in disorganized cellar activity. So far, the truncated form was found only in tumor cells. It suggests that the truncated MK will be used as a tumor marker. It will be interesting to examine whether the appearance of alternatively processed MK mRNA is related to a phenotype of the tumor cells. In case of Wilms' tumor cells, alternative splicing of WT1 cDNA is considered to be a cause of the tumorigenesis (9).

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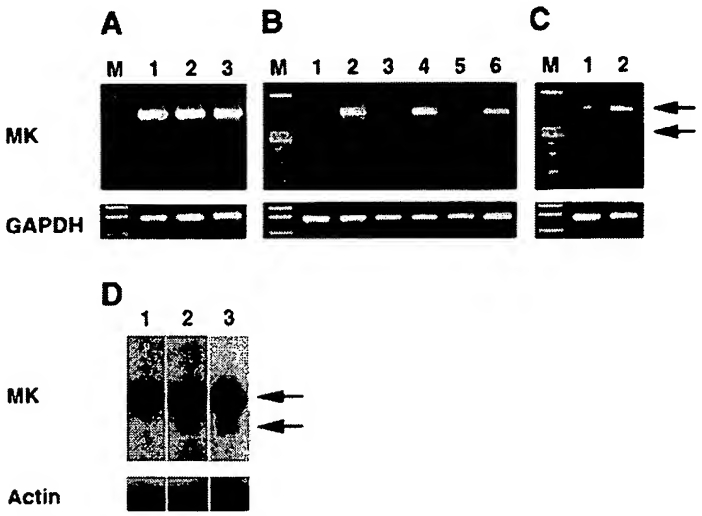


**FIG. 4.** Expression of the mature form and truncated form of MK in tumors and non-tumorous tissues. Arrows indicate the mature form of MK cDNA and the truncated form. MK expression was detected by RT–PCR (A, B, C) and Northern blots (D). A and D, MK expression in gastric carcinoma. Lane 1, gastric carcinoma (origin); lanes 2, 3, gastric carcinoma (lymph node metastasis). A and D are the same sample, respectively. The Northern data were in good agreement with the RT–PCR data. B, pancreatic tumor. Lanes 1,3,5, non-tumorous tissues of pancreas; lanes 2,4,6, pancreatic carcinoma. Lanes 1 and 2, 3 and 4, and 5 and 6 are the same individuals of each. C, Wilms' tumor. Lane 1, non-tumorous tissue of the kidney; lane 2, Wilms' tumor. Both tissues are the same individual. The truncated form of MK was observed lanes A-2,3, B-2,4, C-2, and D-2,3.

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